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ABSTRACT

Cyclin-dependent kinases (CDKs) have an established role in metazoans and yeast in DNA replication, transcription and cell cycle regulation. Several CDKs and their effectors have been identified in the malaria parasite *Plasmodium falciparum* and their biological functions are beginning to be investigated. Here we report results from the functional characterization of Pfmrk and its effector PfMAT1. We validated the interactions between Pfmrk and PfMAT1 and pinpointed their intracellular location. Co-immunoprecipitation studies demonstrated physical interaction between the two proteins and identified the C-terminal domain of PfMAT1 as the Pfmrk activator domain. Immunofluorescence analyses using GFP and RFP-tagged versions of Pfmrk and PfMAT1, respectively, demonstrated the co-localization of these two proteins to the parasite nucleus. Bacterial two-hybrid screen of a *P. falciparum* cDNA library using Pfmrk as the bait identified two plasmodial DNA replication proteins, PfRFC-5 and PfMCM6, as interactors with Pfmrk. We demonstrate that that these two proteins are substrates of Pfmrk-mediated phosphorylation and that PfMAT1 confers substrate specificity to the Pfmrk kinase complex. Collectively, these data suggest a role for Pfmrk in the nucleus of the parasite presumably in regulation of the DNA replication machinery.

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1. Introduction

Cyclin-dependent kinases are a large group of highly conserved proteins that play a critical role in the regulation of various cellular processes such as cell cycle progression, transcription, DNA replication and repair, apoptosis and differentiation [1]. Active forms of CDKs are heterodimers comprising of a catalytic Ser/Thr kinase subunit and a regulatory subunit, usually the cognate cyclin. Binding of a CDK with its cognate cyclin results in the displacement of a sterically hindering "T-loop" and exposure of residues in the catalytic cleft resulting in a partially active enzyme. The action of CDK activating kinase (CAK) complex then leads to complete activation of the CDK-cyclin complex via phosphorylation of a conserved Thr residue within the "T-Loop" [2]. In metazoans, CDK7 functions as a CAK which phosphorylates and activates cell cycle CDKs. The CAK complex, which is also involved in the process of transcription, consists of CDK7, cyclin H and a third stabilizing partner MAT1 [1]. The MAT1 protein stabilizes and influences the substrate specificity of the CDK7 kinase complex [3]. Sequence analysis and biochemical data have revealed that the MAT1 protein can be divided into three domains with discrete functions: an N-terminal RING finger domain, a central coiled-coil domain and a hydrophobic C-terminal domain [4]. The C-terminal domain interacts with the CDK7–cyclin H complex and stimulates CDK7 kinase activity. The central domain is involved in anchoring the CAK to the TFIIH core, and the N-terminal domain is involved in CTD phosphorylation and transcription activation [4]. The CAK complex has been shown to be localized in the nucleus and its substrates are predominantly nuclear [5,6].

Several of these familiar components of the cell cycle regulatory machinery have been identified and characterized in the malaria parasite *Plasmodium falciparum* [7]. The plasmodial life

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cycle is characterized by alternation between proliferative and non-proliferative stages. Sporozoites injected into the bloodstream by an infected *Anopheles* mosquito migrate to the liver and invade hepatocytes, producing up to 40,000 merozoites per hepatocyte. These merozoites are then released into the bloodstream after lysis of the infected hepatocyte, and in turn invade erythrocytes, yielding 20–32 daughter merozoites per erythrocyte, which upon release go on to invade more erythrocytes [8]. Although the distinct phases of the malaria cell cycle have not been defined, it is generally accepted that the invading merozoite is in the G1 stage of the cell cycle. At 18 h post-invasion, the ring stage parasite progresses to trophozoite stage and DNA synthesis is thought to be initiated. Several rounds of asynchronous nuclear division ensue the trophozoite stage [9]. The exact molecular mechanisms involved in the regulation of this atypical cell cycle are poorly understood [10], however they are being actively investigated for identification of potential novel drug targets.

Several CDKs, cyclins, and a CDK effector protein have been identified in *P. falciparum* using homology-based PCR and database mining approaches [10]. Pfmrk shares 46% sequence identity with human CDK7 and the recombinant protein phosphorylates RNA Pol II CTD and Histone H1 *in vitro* in the presence of either human cyclin H or Pfcyc-1 [11–13]. PfMAT1, a putative homolog of human MAT1, stimulates the CTD kinase activity of Pfmrk in a cyclin-dependent manner *in vitro* [14]. Although it has been demonstrated that plasmodial CDKs are amenable to inhibitory mechanisms exerted by mammalian CDK inhibitors [15], to date CDK inhibitory proteins have not been identified in *Plasmodium*. Therefore, the regulatory mechanisms for activating and inhibiting CDK activity in *Plasmodium* remain to be unraveled. Furthermore, the substrates of CDKs and how CDKs regulate the malarial cell cycle are not known.

Pfmrk displays highest similarity to mammalian CDK7; however it is unclear if Pfmrk is a functional homologue of CDK7 [13]. In metazoans, CDK7 has a dual function as a CAK and as the kinase element of the general transcription TFIIH complex which phosphorylates the CTD of RNA polymerase II. In *Saccharomyces cerevisiae*, the CDK7 ortholog Kin28 is a CTD kinase and a member of the TFIIH complex but does not possess CAK activity. In *Schizosaccharomyces pombe*, two CAKs have been found—the Mcs6 complex and the Csk1 [16]. Hence, CDK7 orthologs from various metazoans seem to have different biological functions. Therefore, Pfmrk may have a unique role in the cell cycle that may have arisen through *Plasmodium* evolution. In fact, phylogenetic analyses suggested that Pfmrk may not belong to the CDK7 family of CDKs [17,18]. Previous biochemical studies suggested that Pfmrk in association with Pfcyc-1 or Pfcyc-1-PfMAT1 does not possess CAK activity towards the CDK1 ortholog PfPK5 [12,14]. Although Pfmrk may function as a CAK in association with different cyclin subunits or towards different plasmodial CDKs, initial inquiries were raised as to the biological function of Pfmrk in the absence of a *bona fide* CAK activity.

The process of DNA replication is initiated by the action of many enzymes [19]. First, the origin–recognition complex (ORC) is assembled at the replication origin. This is followed by the recruitment of the pre-replication complex (pre-RC) consisting of Cdc6, Cdt1and MCM proteins to the ORC. The MCM2–7 proteins form a hexameric complex that assembles at replication origins during early G1 phase [20]. Coordination of the functional interactions between the MCM2–7 proteins and other components of the pre-RC is mediated by CDKs [19,21]. Subsequently, loading of DNA polymerase occurs and is mediated by the replication factor complex (RFC) [22]. The RFC consists of five members (RFC-1, RFC-2, RFC-3, RFC-4 and RFC-5). The loading and activation of the pre-RC occurs at late G1/early S-phase. Once DNA replication is initiated, the Pre-RC is disassembled thus preventing the re-replication of DNA until the cell cycle resets again—in the subsequent G1 phase. Sequential activation and deactivation of CDKs and other cell cycle regulatory kinases, ensure that the replication of DNA only occurs once per cell cycle [23].

Another malarial CDK, PfPK5, has been proposed to play a role in DNA replication [24,25]. PfPK5 expression and kinase activity levels peak 36 h post-invasion and PfPK5 was shown to co-localize with DNA in the early trophozoite stages, i.e., before or at the onset of DNA synthesis. Two lines of evidence suggest a role for PfPK5 in the S-phase (a) elevated PfPK5-associated kinase activity was detected in parasites treated with the DNA synthesis inhibitor aphidicolin and (b) treatment of parasites with the PfPK5 inhibitors flavopiridol and olomoucine results in decreased DNA replication [26]. Since no obvious NLSs could be detected in the PfPK5 sequence, the possibility exists that it is co-transported into the nucleus along with its cognate cyclin (or other effector) where it regulates DNA replication. Nuclear import/export of CDKs and cyclins has been demonstrated to be a mechanism to regulate cell cycle progression [27].

In this study, we describe the functional characterization of the *P. falciparum* CDK Pfmrk. We have validated its interactions with PfMAT1, demonstrated co-localization to the nucleus and identified components of the DNA synthesis machinery as substrates of this enzyme. Taken together, these results suggest a role for Pfmrk in the DNA replication process.

2. Materials and methods

2.1. Molecular cloning of PfMAT1 deletion mutants

DNA for the construction of PfMAT1 deletion mutants was amplified from a plasmid template of PfMAT1 full-length cDNA in pQE30 [14] with oligonucleotides containing a *BamHI* restriction site. The full-length and truncated PfMAT1 PCR products were digested with *BamHI* (Promega) and cloned into the *BamHI* site of pET15b (Novagen). All the constructs were sequenced to check for orientation and correct reading frame.

2.2. Molecular cloning of MBP-PfMAT1

The MBP-tagged version of PfMAT1 was constructed by amplifying PfMAT1 cDNA with oligonucleotides containing *EcoRI* and *HindIII* restriction sites and cloning the PCR product into pMAL-c2X vector (New England Biolabs).

2.3. Expression and purification of recombinant proteins

Pfmrk (PF10_0141), Pfcyc-1 (PF14_0605), PfMAT1 (PFE0610c), and RNA Pol II CTD were expressed and purified from *Escherichia coli* as GST tagged (Pfcyc-1 and CTD) or 6 × His tagged (Pfmrk and PfMAT1) fusion proteins as previously reported [12,14]. PfRFC-5 was expressed in BL21-CodonPlus-RIL *E. coli* cells, and purified over a nickel chelating column under identical conditions previously described for PfMAT1 [14]. PfRFC-5 purified as a 40 kDa protein as determined by SDS-PAGE. GST-tagged PfMCM6 was purified on a GSTrap HP column (GE Healthcare) using the same protein purification procedures as described for Pfcyc-1. GST-PfMCM6 purified as a110 kDa protein as determined by SDS-PAGE. MBP-PfMAT1 protein was purified on an amylose column (New England Biolabs) according to the manufacturer's instructions (www.neb.com).

2.4. Kinase assays

Kinase assays were performed in filter bottom microtiter plates as previously described [14]. Briefly, 1.5 μg of Pfmrk was assayed in a 50 μl kinase reaction containing kinase buffer (50 mM Tris–HCl pH 7.5, 2.5 mM $MnCl_2$, and 1.0 mM DTT) supplemented with 3.0 μg Pfcyc-1 and various concentrations of substrates. The reaction was started by the addition of an ATP mix containing 12 μM ATP (Sigma)

and 5 μCi (γ-^{32}P) ATP, 3000 Ci/mmol (Amersham) and activity assayed at 30 °C for 30 min. Plates were washed on a vacuum manifold (Whatman) with 5% phosphoric acid. Following the wash, scintillation fluid was added to each well and activity measured in a Topcount microtiter plate scintillation counter (Packard). Phosphorylation of PfMCM6 was determined using a Kinase-Glo assay system (Promega) with the same assay conditions as above. Each assay was performed in triplicate and average kinase activity plotted as relative kinase activity as a percentage above background (kinase alone without substrate). For autoradiography, kinase reactions as described above were assayed under identical conditions described, however, following the incubation time, the reaction was stopped by the addition of 4 × SDS loading buffer and boiled. The reactions were resolved on a 12% SDS-PAGE, transferred to nitrocellulose, and exposed to X-ray film.

2.5. Pull-down assays

2.5.1. Detection of proteins bound to immobilized GST-Pfcyc-1

10 μg of GST-Pfcyc-1 was incubated with 500 μL of immobilized glutathione (Pierce) previously pre-incubated with Wash Buffer (1:1 of 1 × PBS:ProFound Lysis Buffer from Pierce) at 4 °C on a rotary shaker for 1 h. The sepharose beads were then washed five times with 1 mL Wash Buffer and incubated with 5 μg of various proteins at 4 °C on a rotary shaker for 1 h. Following incubation, the beads were washed five times with Wash Buffer and boiled with 2 × SDS-PAGE gel loading buffer [28] for 5 min and subjected to SDS-PAGE. Western Blotting was performed using PVDF membranes (Pierce) according to the manufacturer's instructions. Anti-His-HRP (Pierce) at 1:5000 and anti-MBP-HRP (New England Biolabs) antibodies at 1:25,000 were used to detect Pfmrk and PfMAT1, respectively.

2.6. Detection of proteins bound to immunoprecipitated MBP-tagged PfMAT1

For co-immunoprecipitation reactions, 5 μg of each protein in the reaction was allowed to incubate on ice for an hour with frequent mixing. The proteins were then incubated with 500 μL anti-MBP-antibody (New England Biolabs) previously diluted to 1:5000 in Co-IP buffer (50 mM Tris pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40) at 4 °C on a rotary shaker for 1 h. 100 μL of a 50% slurry of Protein A-agarose (Sigma) resuspended in Co-IP buffer was added and incubated at 4 °C on a rotary shaker for 1 h. The beads were then washed five times with 1 mL Co-IP buffer and boiled with 2 × SDS-PAGE gel loading buffer for 5 min and subjected to silver staining (Pierce).

2.7. Creation of GFP-tagged Pfmrk and RFP-tagged PfMAT1 constructs

In order to construct the pCHDR–Pfmrk-GFP (the HPG plasmid), Pfmrk cDNA [14] was first cloned into the pENTR/D-TOPO vector to create the Pfmrk–pENTR plasmid according to the instructions outlined in the Invitrogen GATEWAY manual (www.invitrogen.com). Pfmrk–pENTR, the GFP tag in the pDONRP2R-P3 vector, the HSP86-5′promoter in pDONRP4-P1R, and the pCHDR-3/4 destination vector [29] were combined in an LR reaction (according to protocols outlined in the GATEWAY manual from Invitrogen). In order to construct the pHBIL–PfMAT1-RFP (the HMR plasmid), the PfMAT1 cDNA was cloned into the pENTR/D-TOPO vector to create the PfMAT1–pENTR plasmid. The PfMAT1–pENTR plasmid, the RFP tag in the pDONRP2R-P3 vector, the HSP86-5′promoter in pDONRP4-P1R, and the pHBIL-3/4 destination vector [29] were combined in another LR reaction. The recombinant plasmids were propagated in *E. coli* and extracted by Maxi-prep (QIAGEN) columns for transfection of *P. falciparum* cultures.

2.8. Transfection of P. falciparum

Synchronized *P. falciparum* strain W2 parasites cultured in complete medium at 5% hematocrit and 7% parasitemia (predominantly rings) were used for transfection. Prior to electroporation, a 5 ml infected red blood cell (RBC) suspension was centrifuged at 1500 g at 4 °C and placed on ice. About 100 μg of plasmid DNA dissolved in 200 μL of 2 × sterile PBS was mixed with the 400 μl of the cell suspension in a pre-chilled 0.2 cm cuvette. Electroporation was performed in a BTX electroporation system at 320 V, 186 Ω resistance and 950 μF capacitance. Parasites were first transfected with pCHDR–Pfmrk-GFP (HPG) and cultured in 25 mL culture flasks for 48 h without drug selection. Thereafter, the selection drug WR992210 was introduced in the culture medium at 2.5 ng/mL. Drug resistant, GFP-fluorescing parasites appeared in 21–25 days post-transfection. These Pfmrk-GFP expressing parasites were then transfected with pHBIL–PfMAT1-RFP (HMR) and selected for BSD at 2 ng/mL. Drug-resistant parasites were established after 2 weeks of continuous culture and maintained on dual drug (WR99210 and BSD) selection for downstream microscopy studies.

2.9. Fluorescence microscopy on Pfmrk-GFP and PfMAT1-RFP parasites

Infected RBCs were centrifuged and washed twice with 1 × ice-cold PBS. The pelleted RBCs were then stained with Hoescht 33258 (Invitrogen, 1 mg/mL in ice-cold PBS) for 20 min in the dark on a low-speed shaker. The RBCs were pelleted once again, washed twice with ice-cold 1 × PBS, pelleted and placed on to microscope slides with cover slips. The parasites were visualized for the fluorescence generated by Hoescht, GFP and RFP in an Olympus BX51 fluorescence microscope and the images were captured with a QImaging RETIGA-2000RV FAST 1394 digital camera using the QImagePro software.

2.10. BacterioMatch two-hybrid screen

The expression of the highly conserved malarial CDKs is toxic to yeast (Personal Communication, Doug Lacount). Therefore, we chose to employ a bacterial two-hybrid screen to identify proteins that interact with Pfmrk rather than the more frequently used yeast two-hybrid system. A bacterial two-hybrid system (BacterioMatch II, Stratagene) was used to identify regulatory proteins and substrates of Pfmrk. We constructed a two-hybrid cDNA library from *P. falciparum* strain 3D7 cultures consisting of 80% trophozoites and 20% rings and schizonts. The cDNA library was size fractionated from 400 bp to 3 kb and cloned into the pTRG target vector according to the manufacturer's instructions (Statagene). Pfmrk served as the bait in the screen and was cloned into the *EcoRI* and *BamHI* sites of the pBT vector according to the manufacturer's protocol.

After screening for histidine auxotrophy and streptomycin resistance, several positive clones were selected. These clones were sequenced and a BLAST [30] analysis was performed against the plasmodial genome database (Plasmodb.org). Results from our BLAST search yielded several proteins that have been shown to interact with CDKs in other organisms (Table 1). All two-hybrid interactions were verified in at least three independent clones. From our list of four two-hybrid hits, we selected the already characterized mini chromosome maintenance protein-6 (PfMCM6) (PF13_0291) [31] and the replication factor complex subunit five (RFC-5) (PF11_0117) for further analyses. Full length PfRFC-5 and PfMCM6 were subsequently cloned into the pTRG vector via RT-PCR and their interactions with Pfmrk were confirmed through a positive validation two-hybrid screen (Stratagene).

Table 1
List of proteins found to interact with the "Pfmrk bait" in the bacterial two-hybrid system.

PlasmoDB ID	Gene name	Amino acid sequence of the insert	Implicated in biological process	Identifiable motifs
PF13_0291	PfMCM6	GALMYADQGICCIDEFDKMDEKDRVAIHEAMEQQTISITKAGI-QATLNARASVLAACNPKYGRYDTLKTFAQNVNIPA	DNA replication	DNA-dependent ATPase
PF11_0117	PfRFC-5	SKTPMYRIFVFKDAEFLSEGAQAGLRRTLETYIRNARVILHLE-HLSKIIEPLKSRCICIRVPLPSEEEIYSVLQNICKQE	DNA replication	ATP/GTP binding
PF11_0232	Hypothetical Protein	KDNIYACCHIELSTPYLVKAYINVPC	Unknown	Cyclin binding
PF07_0126	Hypothetical Protein	RERAVSCRKQAEKLFNLPEIQPRNRWNQIKVNGTSHIKKAA-KLPRCEGIGYDELSQSWVSTFVVHKKF	Transcription	AP2 DNA binding

2.11. Cloning of PfMCM6 and PfRFC-5

PfMCM6 was amplified by polymerase chain reaction from *P. falciparum* strain 3D7 cDNA using forward primer 5′-GGGAATTCTCAGCTATATTTAATGAAAGTGAATTATC-3′ and reverse primer 5′-GGGAATTCTTAAAAATTGTCAATTTC TTCTTG-3′, digested with *EcoRI* and cloned into pGEX-5X-3 (GE Healthcare). This cloning step incorporated a GST tag at the 5′ end of the PfMCM6 gene. PfRF-C5 was amplified by polymerase chain reaction from *P. falciparum* strain 3D7 cDNA using forward primer 5′-GGGGATCCATGTGGCTTGAAAAATATAGC-3′ and reverse primer 5′-GGCTGCAGTTATTTTCCATGTAAATTATATTTC-3′. This step facilitated subcloning of PfRFC-5 into the *BamHI* and *PstI* sites of the pQE-30—the 6 × His tagged expression vector (Qiagen).

3. Results

3.1. The C-terminal hydrophobic domain of PfMAT1 is sufficient to activate Pfmrk–Pfcyc-1 kinase activity

The PfMAT1 protein sequence displays an overall sequence similarity of 49% to human MAT1 [14]. Human MAT1 contains several domains responsible for its diverse biological functions; in particular, the C-terminal domain stimulates CDK7 activity [4]. In order to determine if the analogous domain of PfMAT1 is involved in activating Pfmrk, we created truncated versions of PfMAT1 and investigated their ability to activate Pfmrk together with Pfcyc-1 in an *in vitro* kinase assays (Fig. 1A). The C-terminal region of PfMAT1 comprising of the hydrophobic domain (amino acids 150–260)

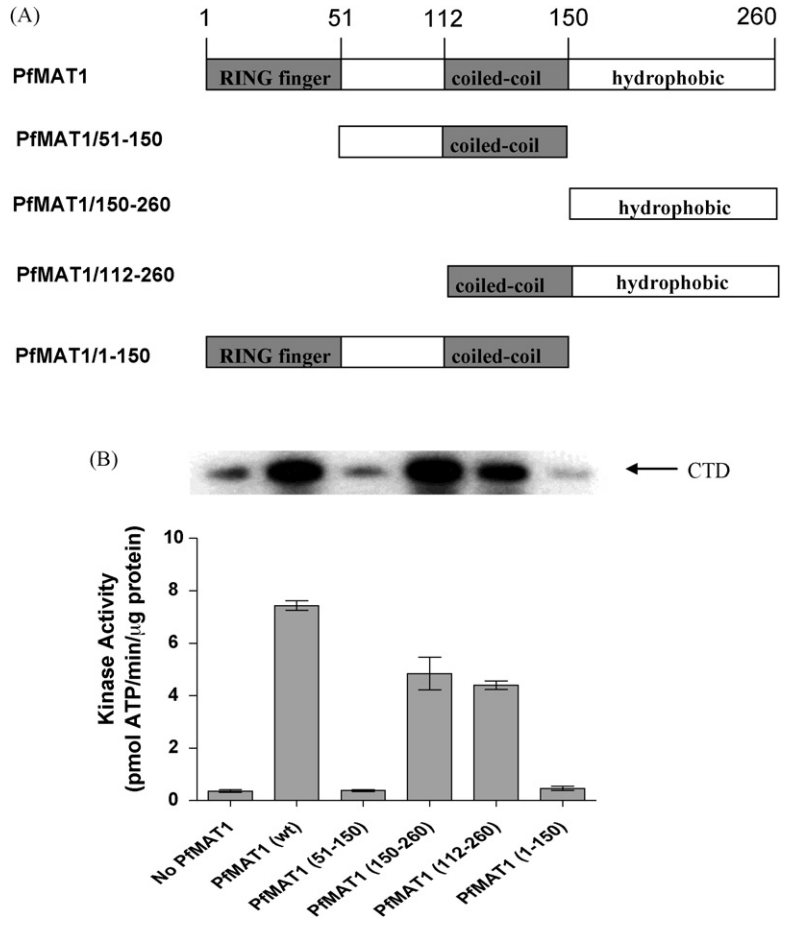

Fig. 1. (A) Schematic drawing of PfMAT1. The ring finger domain (1–51), the coiled-coil domain (112–150) and hydrophobic domain (150–260) are shown. N- and C-terminal truncations (PfMAT1/51–150, PfMAT1/150–260, PfMAT1/112–260 and PfMAT1/1–150) are represented. (B) The hydrophobic C-terminus of PfMAT1 stimulates Pfmrk kinase activity. The ability of PfMAT1 truncated mutants to activate Pfmrk activity was evaluated. Kinase reactions containing 1.5 μg of Pfmrk, 3.0 μg Pfcyc-1 and 2.0 μg CTD were assayed in the presence of 1.5 μg of full-length and various PfMAT1 truncated mutants. Pfmrk kinase activity and the extent of CTD phosphorylation was determined by microtiter plate scintillation measurements and autoradiography, respectively.

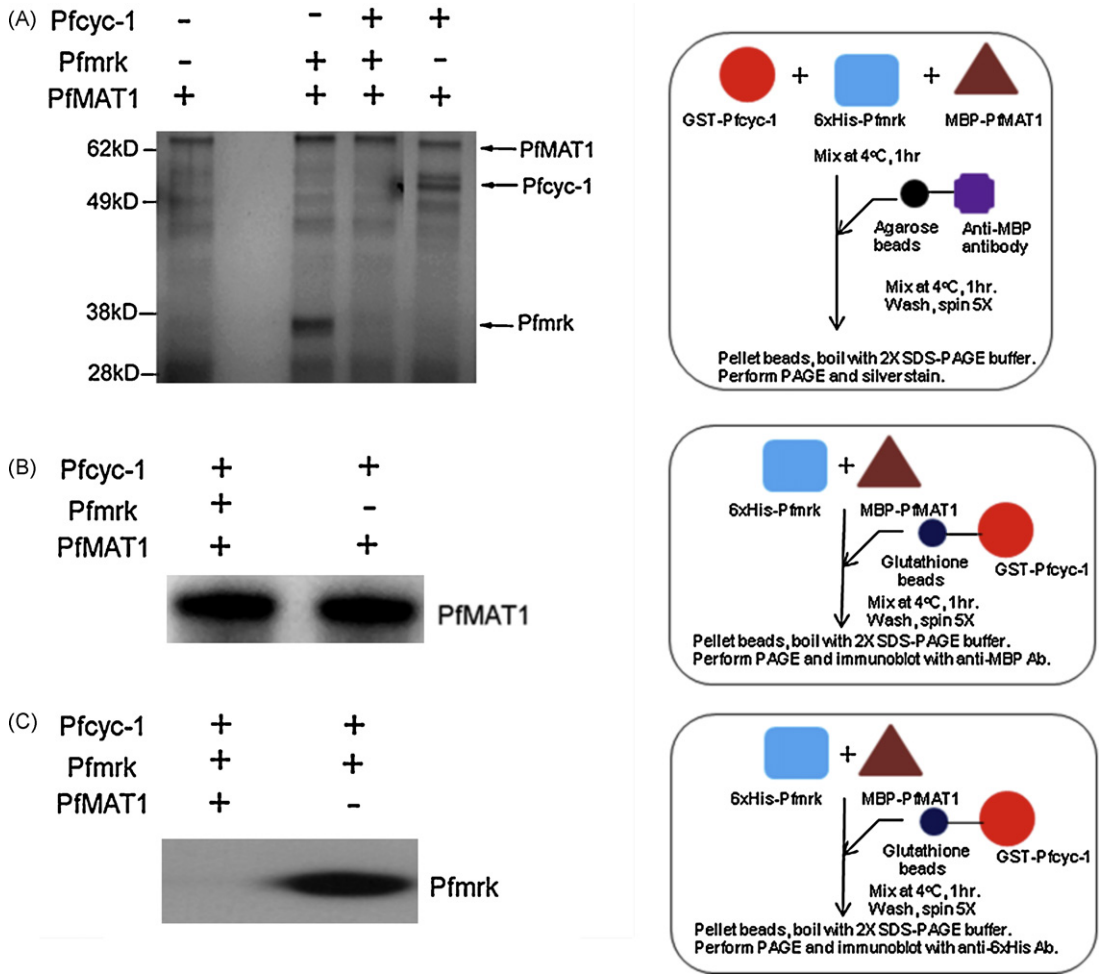

Fig. 2. (A) PfMAT1 binds to Pfmrk and Pfcyc-1. Various proteins (MBP-tagged PfMAT1, 6 × His-tagged Pfmrk and GST-tagged Pfcyc-1) in the reaction were mixed, immunoprecipitated with anti-MBP antibody bound to protein A-sepharose beads, subjected to SDS-PAGE and silver stained. Arrows identify PfMAT1, Pfcyc-1and Pfmrk. (B) PfMAT1 binds to immobilized Pfcyc-1 in the presence and absence of Pfmrk. PfMAT1 (MBP-tagged) and Pfmrk (6 × His-tagged) were allowed to bind Pfcyc-1 (GST-tagged) bound to immobilized glutathione beads. Immunoblot analysis was performed with anti-MBP-HRP in order to detect MBP-PfMAT1. (C) Pfmrk binds to Pfcyc-1 only in the absence of PfMAT1. PfMAT1 (MBP-tagged) and Pfmrk (6 × His-tagged) were allowed to bind Pfcyc-1 (GST-tagged) bound to immobilized glutathione beads. Immunoblot analysis was performed with anti-His-HRP in order to detect Pfmrk.

was sufficient to stimulate Pfmrk activity 4–5-fold (Fig. 1B). The segment of PfMAT1 lacking the C-terminal hydrophobic region, however, failed to stimulate Pfmrk activity (Fig. 1B). Hence, we conclude that the Pfmrk activation domain lies in its hydrophobic C-terminus.

3.2. PfMAT1 physically interacts with Pfmrk and Pfcyc-1

In order to examine the nature of interactions between PfMAT1, Pfmrk and Pfcyc-1, pull-down assays were performed as follows. MBP-PfMAT1 was mixed with Pfmrk in the presence or absence of Pfcyc-1, and immunoprecipitated with anti-MBP antibody bound to protein-A-sepharose. The immunoprecipitated proteins were then visualized with silver staining. PfMAT1 was found to bind independently to both Pfmrk and Pfcyc-1 (Fig. 2A). A control experiment consisting of the MBP tag alone instead of MBP-PfMAT1 did not pull down any of the proteins used in the experiment (data not shown).

To further characterize the interactions between these three proteins, GST-tagged Pfcyc-1 (or the control GST tag) was bound to immobilized glutathione and its ability to bind to PfMAT1 and Pfmrk was examined. PfMAT1 was found to bind to immobilized Pfcyc-1 in the presence and absence of Pfmrk (Fig. 2B). Pfmrk, however binds to immobilized Pfcyc-1 only in the absence of PfMAT1 (Fig. 2C). The GST tag did not bind Pfmrk or PfMAT1 (data not shown). These results suggest that Pfmrk, Pfcyc-1 and PfMAT1 can independently interact with each other; however, we were unable to detect a trimeric complex presumably due to our experimental conditions.

3.3. Pfmrk and PfMAT1 co-localize to the nucleus

In an attempt to understand the cellular function of Pfmrk and to assess the nature of its interaction with its effector PfMAT1 *in vivo*, we constructed Invitrogen's GATEWAY-based [29] GFP-tagged version of Pfmrk (HPG) and RFP-tagged version of PfMAT1 (HMR) under the control of the *P. falciparum* Hsp86 promotor (Fig. 3A and B). We resorted to this approach due to the lack of availability of high-quality antibodies to both Pfmrk and PfMAT1. We were unable to use the antibodies generated against Pfmrk (generated against the Pfmrk peptide sequence MENNSTERYIFK) and PfMAT1 (generated against the PfMAT1 peptide sequence CYQNERKKIHEIVKEEG) for our immunofluorescence analyses, since these antibodies failed to identify the corresponding proteins on western blots from parasite protein extracts (data not shown). Hence, in an effort to express tagged versions of these proteins, W2 *P. falciparum* parasites were transfected with the HPG plasmid (Fig. 3A) and transfectants were selected on medium containing WR99210.

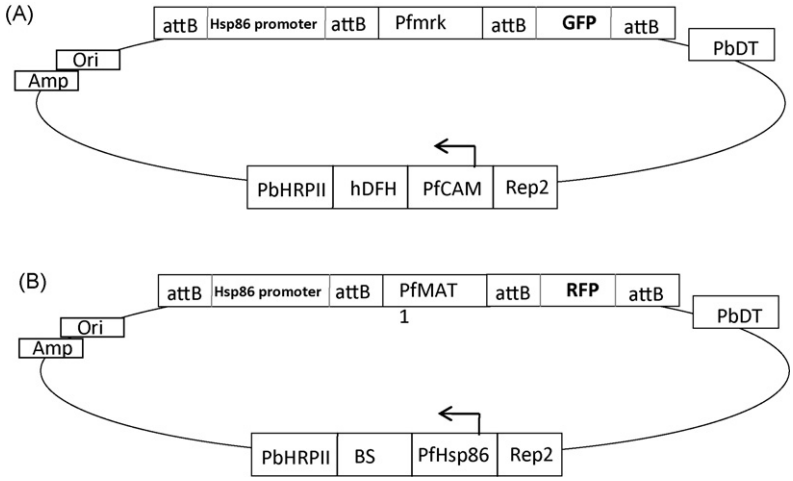

Fig. 3. GATEWAY-based constructs used to transfect parasites. (A) Hsp86 promoter driven Pfmrk-GFP fusion construct (HPG). (B) Hsp86 promoter driven PfMAT1-RFP construct (HMR).

Expression of Pfmrk-GFP fusion protein (68 kDa) in the transgenic parasites was confirmed by immunoblotting using anti-GFP antibodies (Fig. 4A). In order to determine the intracellular location of Pfmrk-GFP, fluorescence microscopic analyses were performed on these transgenic parasites. The intracellular fluorescence pattern of Pfmrk-GFP coincided with nuclear staining observed with the DNA-staining dye Hoechst 33228 suggesting that Pfmrk is localized in the nucleus (Fig. 4B). Next, W2 parasites expressing the HPG plasmid were transfected with the HMR plasmid (Fig. 3B) with the aim of generating parasite cells co-expressing both Pfmrk-GFP and PfMAT1-RFP. Transgenic parasites harboring both plasmids were selected on medium containing both WR99210 and BSD.

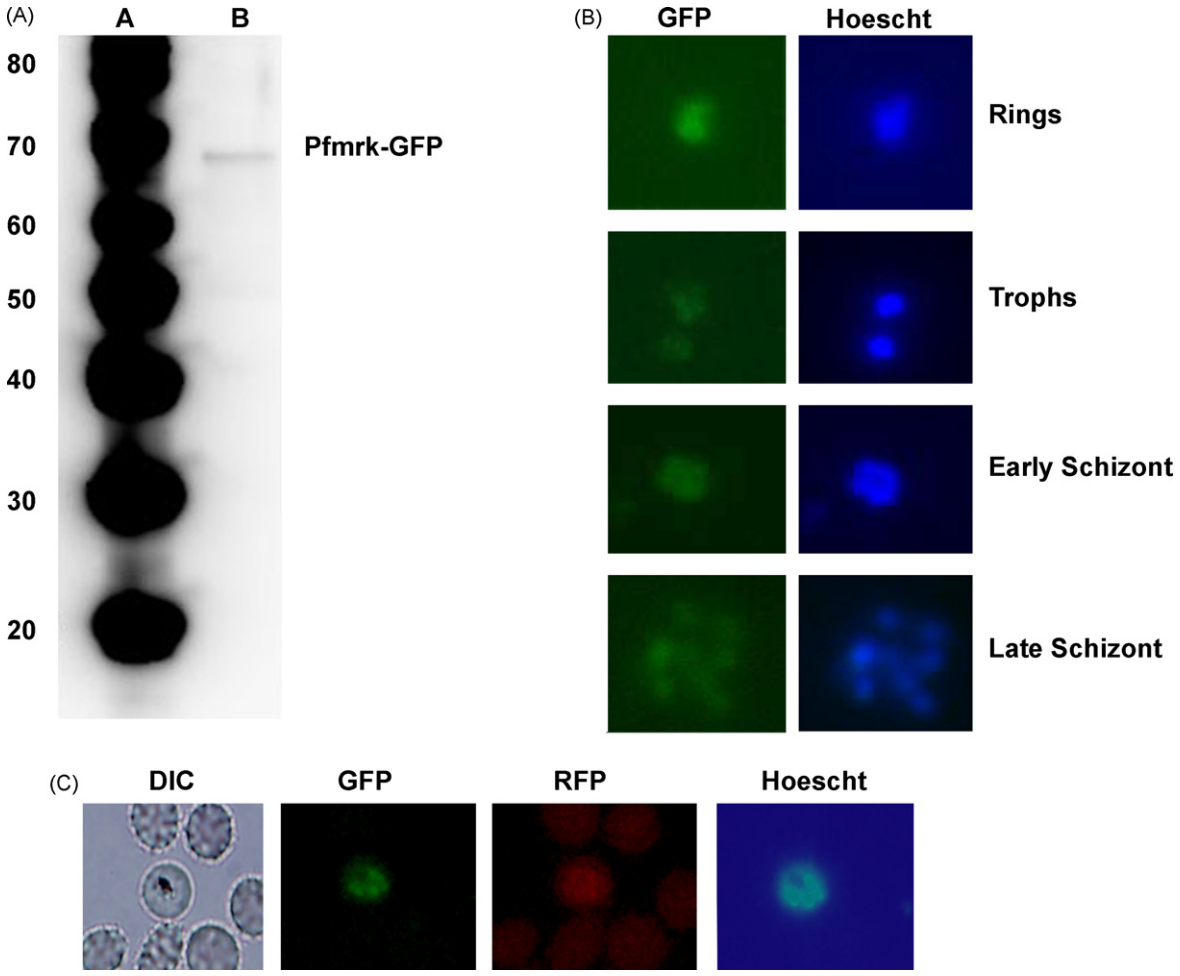

Fig. 4. Analysis of HPG and HPG; HMR transgenic lines. (A) Western blot depicting the expected 68 kDa Pfmrk-GFP protein in crude HPG extracts. (B) GFP and Hoescht fluorescence in ring, trophozoite, early schizont and late schizont stages in the HPG-expressing parasites. (C) GFP, RFP and Hoescht fluorescence observed in schizonts of the HPG and HMR co-expressing parasites.

Fig. 5. CLUSTAL W alignment of RFC-5 subunits from *Drosophila melanogaster* (NM_135650.2), *Schizosaccharomyces pombe* (NP_595646.1) and *Plasmodium falciparum* (PF11_0117). Identical amino acids are depicted in bold. The predicted motifs in PfRFC-5 are indicated in color and highlighted. Residues 40–47 (in red) correspond to the "WalkerA" motif, residues 41–48, 132 and 161 (highlighted) are predicted to fall within the ATP-binding site, residues 128–133 (in purple) correspond to the "WalkerB motif" and residue 175 (in blue) corresponds to the "Arginine finger". The amino acid numbers for PfRFC-5 are indicated to the right. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Microscopic analyses of these double transfectants revealed fluorescence signals in the nucleus predominantly in schizonts (Fig. 4C). This observation suggests that Pfmrk and PfMAT1 are localized to the parasite's nucleus, although the RFP fluorescence is more extended suggesting that PfMAT1 may not be strictly localized in the nucleus. Pfmrk and PfMAT1 expressed from a PfCRT (*P. falciparum* chloroquine resistance transporter) promoter [29] in the same GATEWAY vectors displayed a similar intracellular expression pattern (data not shown). The PfCRT promoter is weaker compared to the PfHsp86 promoter and is considered useful in expressing transgenes that are toxic to parasites when expressed in higher levels and does not necessarily result in nuclear localization of transgenes [29]. Hence, it is not unreasonable to conclude that the nuclear localization of Pfmrk and PfMAT1 observed in the dual HPG, HMR lines is a representation of their true native state. Interestingly, we observed the GFP signal for Pfmrk throughout the different intraerythrocytic developmental stages of the parasites, however for PfMAT1-RFP, we were only able to observe the RFP signal in the schizonts. This suggests that PfMAT1 protein stability might be cell cycle-stage dependent.

3.4. *PfRFC-5 and PfMCM6 interact with Pfmrk in the bacterial two-hybrid system*

To date little is known about the *in vivo* substrates of malarial CDKs. Hence, in order to identify substrates of Pfmrk, we performed a bacterial two-hybrid screen on a cDNA library prepared from *P. falciparum* using Pfmrk as the bait. Four proteins PF13_0291 (PfMCM6), PF11_0117 (PfRFC-5), PF11_0232 (hypothetical protein) and PF07_0126 (hypothetical protein) were found to interact with Pfmrk (Table 1). Of these, we chose to focus on PfRFC-5 and PfMCM6 for further investigations. PfRFC-5 displays an overall sequence similarity of 30% to replication factor complex subunits from Drosophila and yeast (Fig. 5). The RFC (replication factor complex) is a complex of five subunits highly conserved in all eukaryotes. RFC loads the "sliding clamp" PCNA (proliferating cell nuclear antigen) onto DNA during replication. The loading of PCNA onto DNA by RFC is an important step in DNA replication and increases the processivity of DNA polymerase [32]. The RFC subunits share eight regions of high sequence similarity that include regions involved in protein complex formation, DNA binding, PCNA binding and DNA replication [32]. The motifs identified in PfRFC-5 when queried on the NCBI Entrez website (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi) include (i) an ATP-binding site encompassing residues 41–48 and residues 132 and 161, (ii) the WalkerA motif {consensus sequence GxxGxGK(S/T)} encompassing residues 40–47, (iii) the WalkerB motif consensus {contains four aliphatic residues followed by two negatively-charged residues}, encompassing residues 128–133 and (iv) an Arginine finger, residue 175 (Fig. 5). RFC proteins belong to the AAA^+ superfamily of ATPases that contain a P-loop NTPase fold. The WalkerA domain forms a loop that binds to the alpha and beta phosphate moieties of the bound nucleotide and the WalkerB motif is important for interacting with Mg^{2+} cation. The Arginine finger is an AAA^+-family specific motif that senses ATP binding and hydrolysis and transmits conformational changes [33]. The RFC clamp loader has been shown to require the Arginine finger sensors (emanating from each of the subunits) to drive DNA binding and loading of the PCNA [34].

Another interactor of Pfmrk, PfMCM6 (*P. falciparum* minichromosome maintenance 6), has been characterized as a member of a hexameric pre-replication complex (pre-RC). The MCM com-

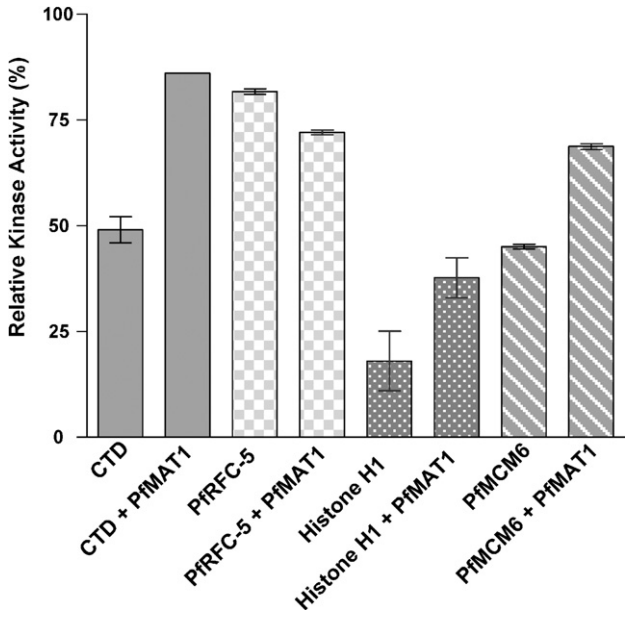

Fig. 6. PfMCM6 and PfRFC-5 are substrates of Pfmrk. Kinase assays were performed with 1.5 μg Pfmrk and 3.0 μg Pfcyc-1 with various substrates of either 2.0 μg CTD, 2.0 μg PfRFC-5, 2.0 μg PfMCM6 or Histone H1 in the absence or presence of 3.0 μg PfMAT1. Each assay was performed in triplicate and average kinase activity plotted as relative kinase activity.

plex has been shown to play a key role in the transition of the pre-RC to the RC and is known to function as a replicative helicase [19]. PfMCM6 was shown to be associated with chromatin at every stage of the parasite's intraerythrocytic life stage [31].

3.5. *Pfmrk phophorylates PfRFC-5 and PfMCM6 in vitro*

Subunits of the RFC and MCM complex are phosphorylated by CDKs and their phosphorylation status might affect their subcellular localization and their ability to bind DNA and other subunits [21,35,36]. To determine whether PfRFC-5 and PfMCM6 are substrates of Pfmrk, *in vitro* phosphorylation assays were performed. Pfmrk was found to phosphorylate both PfRFC-5 and PfMCM6 *in vitro* (Fig. 6). Interestingly, Pfmrk-mediated phosphorylation of PfMCM6 was activated by PfMAT1. In the presence of PfMAT1, Pfmrk kinase activity was stimulated towards the three substrates—CTD, Histone H1 and PfMCM6. PfRFC-5 seems to be a good substrate of Pfmrk, however the addition of PfMAT1 did not stimulate further phosphorylation of PfRFC-5.

4. Discussion

In this study, we have characterized the interactions between Pfmrk and its effector PfMAT1 *in vitro*. The data presented from our co-immunoprecipitation experiments demonstrated stable interactions between PfMAT1 and Pfcyc-1, PfMAT1 and Pfmrk, and between Pfcyc-1 and Pfmrk. The inability to observe a stable trimeric complex (Pfmrk–PfMAT1–Pfcyc-1) is most likely due to our experimental conditions. Co-IP of complexes is sometimes problematic and may reflect stearic hindrance due to the antibodies or immobilizing supports used in the IP. For example, Rossignol et al. [37] observed an interaction between CDK7 and cyclin H, CDK7 and MAT1, but were unable to detect an interaction between cyclin H and MAT1 and Busso et al. [4] demonstrated a weak interaction between cyclin H and MAT1 even though a CDK7–cyclin H–MAT1 exist as an *in vivo* trimeric complex. Although a trimeric complex with Pfmrk was not detected, previous studies demonstrate that PfMAT1 stimulates Pfmrk kinase activity in a Pfcyc-1 dependent manner [14], suggesting the formation of a trimeric complex. The inability to detect the trimeric complex by co-immunoprecipitation using either purified recombinant proteins or parasite extracts (data not shown), may be indicative that future studies to identify the complex may have to utilize affinity chromatography approaches.

Many characteristics of Pfmrk suggest similarities to CDK7. First, Pfmrk activity is stimulated by the binding of cyclin H or Pfcyc-1 (a cyclin with the greatest sequence identity to the CDK7 cognate cyclin H) [12] and the association of PfMAT1 [14]. Second, the CTD of RNA polymerase II is a strong substrate of Pfmrk and third, Pfmrk displays inhibitor sensitivity profiles similar to CDK7 [12,13,38–40]. The present study demonstrates a further similarity to CDK7 by showing that PfMAT1 interacts with both Pfmrk and Pfcyc-1. Interaction with both the CDK and cyclin is believed to stabilize the trimeric complex [41]. The molecular architecture of PfMAT1 is highly similar to human MAT1 with a ring finger, coiled-coil and hydrophobic domain. Analogous to the CDK7 complex, our studies demonstrate that the C-terminal hydrophobic domain of PfMAT1 is responsible for stimulating Pfmrk activity [4]. The coiled-coil and ring finger domains of MAT1 are believed to be involved in protein–protein interactions which may direct the CDK7 complex to specific substrates [3]. In fact, we observe that PfMAT1 influences substrate specificity of Pfmrk. Since the hydrophobic domain of PfMAT1 activates Pfmrk, it is likely that other PfMAT1 domains may be involved in similar PfMAT1–protein interactions as observed with mammalian MAT1.

Immunofluorescence analyses show that Pfmrk localizes in the nucleus along with its effector PfMAT1. This result was surprising since stretches of basic amino acid sequences representing nuclear localization signals (NLSs) [42,43] cannot be detected in the amino acid sequences of Pfmrk. In the case of CDK7, it has been shown that the KRKR motif is required for nuclear targeting and that mutating the sequence to NGER retains the protein in the cytoplasm. This nuclear targeting of CDK7 is necessary to confer the protein with its CAK activity [44]. It is possible that either the NLS signals of these malarial CDKs are cryptic or these proteins are chaperoned into the nucleus by other NLS-containing proteins. Cyclin H is targeted to the nucleus by a C-terminal NLS (KKRKgyedddyvsKKsKh) [45]. Examination of the amino acid sequences of Pfcyc-1 [12], Pfcyc-2, Pfcyc-3 and Pfcyc-4 [46] reveals the presence of putative NLSs in Pfcyc-1 (KKKKNSKKSKKKKLDNK) and Pfcyc-2 (RSNIYNGNK). An NLS has not been identified in the sequences of either hMAT1 or PfMAT1. Hence, it is tempting for us to speculate that Pfmrk may be chaperoned into the nucleus by one of its cognate cyclins or other hitherto unidentified effector(s).

The identification of proteins that interact with Pfmrk provides evidence for a role in distinct cellular processes. Our studies suggest that Pfmrk interacts with proteins believed to be involved in gene expression and DNA replication. One of the hypothetical proteins identified, PF07_0126 is a putative transcription factor. It remains to be investigated if this protein is a transcription factor and whether Pfmrk plays a role in the regulation of gene expression during the *P. falciparum* life cycle. It is not surprising that Pfmrk may have a role in gene expression as CDK7 and orthologs regulate gene expression in association with the basal transcription RNA Pol II TFIIH complex [16,47]. Additionally, we identified an association of Pfmrk with regulators of DNA replication, PfRFC-5 and PfMCM6. In mammalian cells, MAT1 and the CAK complex have been shown to physically interact with the MCM proteins suggesting a role for CDK7 in DNA replication [48]. The interaction with the molecular machinery of DNA replication and transcription is consistent with the nuclear localization of Pfmrk.

Subunits of the RFC and MCM complex are substrates of CDKs [23,49]. In this study, we demonstrated that Pfmrk phosphorylates PfRFC-5 and PfMCM6 *in vitro*. The biological significance of

this phosphorylation is unclear as CDK phosphorylation has been shown to be involved in both activation and deactivation of the DNA replication complexes [50]. The malarial cell cycle operates under the confines of an endoreduplication process known as endomitosis wherein S and M phases alternate without intervening cytokinesis [51,52]. Interestingly, even during endoreduplication (which occurs during differentiation of mammalian trophoblasts and megakaryoblasts), CDKs are involved in regulating the endocyclic process. In fact, differences in CDK regulation are responsible for maintaining either a "normal" mitotic cell cycle versus an endocycle by controlling the assembly and firing of the pre-RC [53]. Many components of the pre-RC and RC are encoded in the plasmodial genome and several have been characterized including MCMs, ORCs, DNA polymerases and PCNA [31,54–58]. Although the *in vivo* phosphorylation status of these DNA replication components remains to be investigated, it is tempting to speculate that Pfmrk-mediated phosphorylation of PfRFC-5 and PfMCM6 may regulate the initiation of DNA replication. The microarray data of Pfmrk, PfMAT1 and Pfcyc-1 suggest high levels of expression from 4 to 28 h peaking at 8 and 24 h—several hours before the start of DNA replication [59]. PfMCM6 protein has been predominantly detected in late trophozoites and schizonts, and gene expression of both PfMCM-6 and PfRFC-5 peaks in late trophozoites through early schizonts—stages where most DNA replication is thought to occur [31,60].

In summary, this study shows that Pfmrk physically interacts with its effector PfMAT1 *in vitro* and that the C-terminal hydrophobic domain of PfMAT1 is the Pfmrk-activating domain. We have also shown that Pfmrk and PfMAT1 co-localize in the nucleus and that regulators of DNA synthesis, PfRFC-5 and PfMCM6, are *in vitro* substrates of Pfmrk. Hence, we suggest that Pfmrk may play an important role in malarial DNA replication. Future studies should be directed toward characterization of the *in vivo* Pfmrk-effector-substrate complexes in an effort to validate and correlate Pfmrk kinase activity with cell cycle mediated DNA replication.

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